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[^{3}H]METHYLCARBAMYLCHOLINE, A NEW RADIOLIGAND FOR STUDYING BRAIN NICOTINIC RECEPTORS*

LEO G. ABOOD† and SUSAN GRASSI

Center for Brain Research, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, U.S.A.

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Abstract—A new radioligand, [^{3}H]methylcarbamylcholine, has been developed for the study of the nicotinic cholinergic and nicotine-like binding sites in rat brain membranes. A Scatchard analysis with the radioligand yielded a K_d of 1.1×10^{-9} M and a B_{max} of 4.0×10^{-14} moles/mg protein which compares with a lower affinity site for (−)-[^{3}H]nicotine having a K_d of 3×10^{-9} M and a B_{max} of 2×10^{-14} moles/mg. Comparable values for the K_d were obtained from a Hill plot and from calculations based on rate constants for association and dissociation. A comparison of the binding affinities of various nicotine analogues, nicotinic cholinergic agents, and other neurotropic agents revealed a close similarity between the two radioligands, with the exception that quaternization of nicotine or carbamate esters increased affinity by at least an order of magnitude with [^{3}H]methylcarbamylcholine and resulted in a comparable decrease in affinity with [^{3}H]nicotine as the ligand. The binding of [^{3}H]methylcarbamylcholine, like [^{3}H]nicotine, was not displaceable by muscarinic cholinergic antagonists. It was concluded that, although [^{3}H]methylcarbamylcholine and [^{3}H]nicotine bind to a common receptor in brain, the functional and chemical characteristics of the receptor(s) differ in some respects from peripheral nicotinic cholinergic receptors.

In the course of investigating the structure–activity relationships of the [^{3}H]nicotine binding sites to rat brain membranes and purified receptor [1], it was observed that carbamylcholine and substituted carbamate esters of choline exhibit a relatively high affinity for the nicotine site [1]. At a concentration of 10^{-9} M *S*-(−)-[^{3}H]nicotine, *N*-methylcarbamylcholine has an IC_{50} value of 8×10^{-9} M as compared to a value of 2×10^{-9} M for unlabeled nicotine [2]. Since carbamate esters of choline are considerably more stable than acetylcholine, a study was undertaken with [^{3}H]methylcarbamylcholine ([^{3}H]MCC) of high radioactive specific activity to determine its similarity to the [^{3}H]nicotine and [^{3}H]acetylcholine binding sites in rat brain membranes. A number of studies with [^{3}H]acetylcholine, prepared enzymatically with [^{3}H]acetic acid, have alluded to the similarity in the receptor binding characteristics of the two ligands [3, 4].

The present study demonstrates the effectiveness of [^{3}H]MCC as a ligand for investigating the nicotine-like and nicotinic cholinergic binding sites in brain tissue.

METHODS

Synthesis of dimethylaminoethyl (DMAE) methylcarbamate and MCC. To 0.05 moles of dimethylaminoethanol in 100 ml of dry toluene was added 0.07 moles of methylisocyanate, and the mixture was refluxed for 16 hr. After removal of the solvent *in vacuo*, the viscous liquid was taken up in 25 ml of $CHCl_3$ and extracted with H_2O. Upon removal of the $CHCl_3$, a white oily product was obtained and the final product was recovered after distillation *in vacuo* at 10 mm and a temperature of 135°. The yield was 85%.

An infrared analysis of DMAE methylcarbamate yielded the following bands:

1730, 1540, 1385, 1100, 960, 995, 790 cm^{-1}

Methylcarbamylcholine was prepared by adding 0.012 moles of methyl iodide to 0.010 moles of DMAE methylcarbamate in 50 ml acetone and allowing the reaction to proceed overnight at room temperature. The white crystalline material was filtered, washed with 50 ml of ethyl ether, and dried. The yield was 98%. Analysis by mass spectroscopy yielded the following fragment with percent relative abundance:

58 (100%), 142 (27%), 28 (21%), 57 (12%), 71 (8%), 42 (8%), 30 (7%), 56 (6%), 59 (6%), 44 (6%)

[^{3}H]MCC was prepared by New England Nuclear by quaternization of DMAE methylcarbamate with [^{3}H]CH_3I. The purity of the [^{3}H]MCC was verified by HPLC.

Measurement of [^{3}H]*MCC and* [^{3}H]*nicotine binding.* The procedure for preparation of rat brain membranes and for measuring specific [^{3}H]MCC and [^{3}H]nicotine binding is described elsewhere [2]. Membranes were obtained from whole rat brain after homogenization in 30 vol. of 0.05 M $NaPO_4$, pH 7.0, and centrifugation at 50,000 *g* for 30 min. To a 2-

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† Address all correspondence to: Dr. Leo G. Abood, Center for Brain Research, University of Rochester School of Medicine and Dentistry, Box 605, 601 Elmwood Ave., Rochester, NY 14642.

ml polypropylene tube was added 2 mg membrane protein along with various concentrations of either [^{3}H]MCC (sp. act. = 80 Ci/mmole) or (−)-[^{3}H]nicotine (New England Nuclear, sp. act. = 75 Ci/mmole) with or without various concentrations of unlabeled nicotine, nicotine analogues, carbamate esters, and other agents, in a final volume of 1.2 ml of 0.05 M $NaPO_4$, pH 7.0. The relationship of pH to the binding of the two ligands was determined with 0.05 M $NaPO_4$ buffer. All assays were performed in triplicate. After incubating in an ice bath (0–4°) for 30 min, the tubes were centrifuged in an Eppendorf centrifuge for 2 min and the pellet was washed twice by filling the tubes with buffer and aspirating. The bottom of the tubes was then cut off (animal nail clipper) and counted by liquid scintillation.

Psychotropic evaluation of various agents. The psychotropic action of the various agents was determined by administering various doses into the fourth ventricle through chronically implanted cannulae, as described elsewhere [5]. A dose of 4 nmoles of (−)-nicotine in 1 μl resulted in prostration of all four limbs, while 2 nmoles (IC_{50}) produced prostration in the hind limbs and some weakness in the forelimbs.

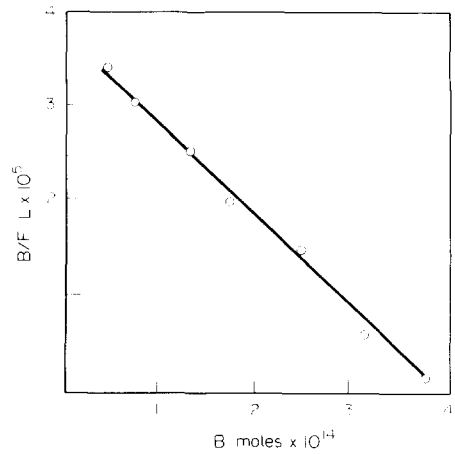

Fig. 2. Scatchard plot of [^{3}H]MCC binding to rat brain membranes. The plot represents a typical experiment from three separate experiments with coefficient of variation of the K_d and B_{max} values being under 8%. A 1000-fold excess of unlabeled ligand was used at each concentration of radioligand to obtain specific binding. B = amount bound and F = concentration of free [^{3}H]MCC.

RESULTS

Comparison of pH curves of [^{3}H]*MCC and* [^{3}H]*nicotine binding.* The pH curves for both [^{3}H]MCC and [^{3}H]nicotine binding had a pH optimum around 6.5 (Fig. 1). Although the shapes of the curves were similar, the change in pH on either side of the optimum was considerably greater for [^{3}H]MCC.

Scatchard analyses of [^{3}H]*MCC binding.* A Scatchard plot of [^{3}H]MCC in the presence of unlabeled MCC was linear, yielding a K_d of 1.1×10^{-9} M and a B_{max} of 4.0×10^{-14} moles/mg protein of whole rat brain membranes (Fig. 2 and Table 1). Replacement of unlabeled MCC with unlabeled (−)-nicotine also yielded a linear Scatchard with comparable K_d and B_{max} values (data not shown). A Hill plot of the data [log $(B/B - B_{max}$ vs log F) yielded a Hill binding constant of 9×10^{-10} M, which is in close agreement with the K_d determined by Scatchard analysis (Table 1).

K_d *calculation from rate constants for association and dissociation.* The rate constant for association of [^{3}H]MCC binding to rat brain membranes was determined to be $3.0 \times 10^5\ M^{-1}\ sec^{-1}$; the dissociation constant was $1.0 \times 10^{-3}\ sec^{-1}$. The K_d, calculated from the ratio of the rate constants, was 3.3×10^{-9} M, a value which is in close agreement with that obtained by Scatchard analysis (Table 1).

Comparison of various agents in competition with [^{3}H]*MCC and* [^{3}H]*nicotine.* A variety of nicotine and cholinergic agents were compared for their abilities to compete with [^{3}H]MCC and [^{3}H]nicotine binding to rat brain membranes (Table 2). At a concentration of 1 nM [^{3}H]nicotine, unlabeled (−)- and (+)-nicotine had IC_{50} values of 3×10^{-9} and 1×10^{-8} M respectively. With either radioligand, increasing the alkyl chain length on the pyrrolidine N resulted in a 3-fold decrease with the *N*-ethyl and a 200-fold decrease in affinity with the *N*-propyl analogues of nicotine. The affinity of *N'*-nicotonium was about three orders of magnitude less than (−)-nicotine with both radioligands. Comparable affinities with both radioligands were observed with the

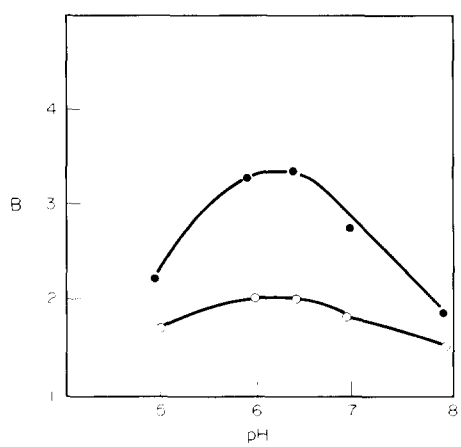

Fig. 1. Binding-pH curves for [^{3}H]MCC (●) and for [^{3}H]nicotine (○). B = moles bound × 10^{-14}.

Table 1. K_d determination by various analytic procedures

Procedure	Data
Scatchard analysis	$K_d = 1.1 \times 10^{-9}$ M $B_{max} = 4.0 \times 10^{-14}$ moles/mg protein
Hill plot	$K_d = 9 \times 10^{-10}$ M
Ratio of rate constants*	$K_d = 3.3 \times 10^{-9}$ M

* Rate constants for dissociation and association of MCC binding to membranes (d/a).

Table 2. Comparison of IC_{50} values for binding of various agents using [^{3}H]nicotine and [^{3}H]MCC as radioligands

	[^{3}H]Nicotine IC_{50} (M)	[^{3}H]MCC IC_{50} (M)	Prostration EC_{50} (nmoles)
(−)-Nicotine	3×10^{-9}	8×10^{-9}	2
(+)-Nicotine	1×10^{-8}	9×10^{-8}	40
N'-Methyl nicotonium	7×10^{-6}	4×10^{-6}	100
N-Ethyl nornicotine	3×10^{-8}	1×10^{-7}	20
N-Propyl nornicotine	6×10^{-7}	1×10^{-6}	80
DMAE methylcarbamate	5×10^{-7}	8×10^{-7}	300
MCC	8×10^{-9}	6×10^{-9}	10
DMAE carbamate	1×10^{-5}	1×10^{-5}	>300
Carbamylcholine	4×10^{-7}	1×10^{-6}	100
Acetylcholine	6×10^{-5}	5×10^{-6}	>100
Hexamethonium	$>1 \times 10^{-4}$	$>1 \times 10^{-4}$	IA
α-Bungarotoxin	$>1 \times 10^{-4}$	$>1 \times 10^{-4}$	IA
QNB	$>1 \times 10^{-4}$	$>1 \times 10^{-4}$	IA
Atropine	$>1 \times 10^{-4}$	$>1 \times 10^{-4}$	IA

To determine psychotropic potency expressed as EC_{50}, at least six rats were used for every agent. DMAE = dimethylaminoethyl; QNB = 3-quinuclidinyl benzilate. The IC_{50} values were determined from plots of various concentrations of agents. The EC_{50} values were based on data from six rats. IA = inactive.

various carbamate esters, MCC having an affinity approaching that of (−)-nicotine. The K_d value for acetylcholine was 5×10^{-5} M, whereas that of hexamethonium, α-bungarotoxin, 3-quinuclidinyl benzilate (QNB) and atropine was greater than 1×10^{-4} M.

Psychotropic action of various agents. The various agents were compared for their abilities to induce prostration following administration into the rat fourth ventricle (Table 2). The (−)-enantiomer of nicotine was 20 times more effective than the (+)-enantiomer and 10, 40 and 50 times more potent than the *N*-ethyl, *N*-propyl, and nicotonium analogues respectively. Among the carbamate esters, MCC had one-fifth the potency of (−)-nicotine, whereas the others were relatively weak or inactive. The remainder of the agents, including acetylcholine, were inactive.

DISCUSSION

The present study has demonstrated that the receptor binding characteristics of [^{3}H]MCC to rat brain are similar to those of [^{3}H]nicotine; however, the Scatchard plot for [^{3}H]MCC was linear whereas that for [^{3}H]nicotine is biphasic [2]. A similar K_d was derived from association–dissociation rate constants and from a Hill plot. The lower affinity site for [^{3}H]nicotine had a K_d of 3×10^{-9} M and a B_{max} of 1×10^{-14} moles/mg membrane protein which compares favorably with the values for [^{3}H]MCC. The higher affinity site seen for [^{3}H]nicotine, with a K_d of 2×10^{-10} M and a B_{max} of 0.5×10^{-14} moles/mg [2], was not seen with [^{3}H]MCC. Although [^{3}H]acetylcholine also appears to have binding characteristics similar to [^{3}H]nicotine [4], [^{3}H]MCC affords the advantages that it is chemically more stable and is more readily prepared. Another similarity between [^{3}H]MCC and [^{3}H]nicotine binding was the pH curve with an optimum of 6.5. A major difference between the two ligands is that [^{3}H]acetylcholine [6], but not [^{3}H]MCC, binding is displaceable by low concentrations of muscarinic antagonists. When muscarinic agonists, such as [^{3}H]-oxotremorine-M [6] and [^{3}H]*cis*-methyldioxolane [7] are employed as radioligands, IC_{50} values are obtainable with muscarinic antagonists in the nanomolar range. Since MCC is closely related to carbamylcholine, which is a muscarinic agonist and readily binds to the muscarinic cholinergic receptor [6], this finding was unexpected.

Although the relative binding affinities of a variety of nicotine analogues and other agents were similar with the two radioligands, there was one striking difference. With either radioligand, quarternization of the pyrrolidine N of nicotine reduced the affinity by three orders of magnitude, whereas quaternization of the carbamate esters resulted in about a 50-fold increase in affinity (compare DMAE methylcarbamate with MCC). It is also noteworthy that the psychotropic potency following intraventricular administration decreased 50-fold with quaternization of nicotine, while increasing over 10-fold after quaternization of DMAE methylcarbamate. The diminished psychotropic potency of *N'*-methyl nicotonium is anomalous in view of the fact that, when administered systemically, it was more potent than nicotine in producing seizures and mortality in rats or mice (unpublished). As discussed elsewhere [1], this difference in the central and peripheral action and receptor affinity of nicotine and *N'*-methyl nicotonium suggests a difference in the nature of the two receptors. Binding studies with [^{3}H]nicotine indicate that there may be at least two, and possibly more, nicotinic sites in rat brain [2, 8], whereas with [^{3}H]MCC only one site is evident, which is presumably similar to the lower affinity site for nicotine. The functional significance of the low and high affinity sites for nicotine is not known. Since 1–2 μl of a 10^{-3} M solution of nicotine administered into the fourth ventricles was required to produce prostration, it appears likely that the lower affinity

site is associated with the psychotropic response. Since MCC and other carbamate esters of alkylaminoalcohols are chemically similar to acetylcholine, it would appear that MCC is interacting with a nicotinic site in brain, while being virtually inactive at a muscarinic cholinergic site.

Insofar as the binding characteristics of [^{3}H]nicotine resemble those of [^{3}H]MCC, both ligands evidently bind to the same receptor. The extremely low affinity of acetylcholine [four orders of magnitude less than (−)-nicotine and MCC] and its correlative lack of psychotropic activity, when supersaturating concentrations of acetylcholine are administered intraventricularly, leave partly unexplained the functional and biochemical nature of the sites. A similar disparity between the binding affinity and function of acetylcholine has been observed in the *Torpedo* electric organ which exhibits both a low and high affinity site for the nicotine antagonist α-bungarotoxin [9]. Presumably, occupancy of the low affinity site by acetylcholine activates the ionic channel whereas occupancy of the high affinity site leads to desensitization, so that a nicotinic antagonist may prevent channel activation without occupying the recognition site [10]. It is possible that either the higher affinity site involves a desensitized cholinergic receptor or that MCC and nicotine function as agonists at a high affinity recognition site whose relationship to acetylcholine remains obscure.

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